Inhibition of protein kinase Cbeta prevents foam cell formation by reducing scavenger receptor A expression in human macrophages

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Abstract: BACKGROUND: Low-density lipoprotein (LDL) uptake by monocyte-derived macrophages is a crucial step in foam cell formation and early atherosclerotic lesion. Increasing evidence supports the theory that activation of protein kinase Cbeta (PKCbeta) is involved in many mechanisms promoting atherosclerosis. Thus, we investigated whether inhibition of PKCbeta prevents foam cell formation. METHODS AND RESULTS: The differentiation of human primary monocytes or the monocytic THP-1 cell line into monocyte-derived macrophages was induced by phorbol 12-myristate 13-acetate (PMA; 0.1 mmol/L), a potent activator of PKC. Incubation of monocyte-derived macrophages with DiI-modified LDL (acetylated LDL and oxidized LDL, 10 mug/mL) led to lipoprotein uptake. Interestingly enough, the nonselective inhibitor of PKCbeta(1) and PKCbeta(2), LY379196 (5x10(-7) to 10(-5) mol/L), blunted LDL uptake in monocyte-derived macrophages as shown by flow cytometry. Specific siRNA-mediated knockdown of PKCbeta exerted a similar effect. Furthermore, PMA alone and in the presence of modified LDL induced scavenger receptor A mRNA and protein expression, which was abolished by LY379196. CGP53353, a selective inhibitor of PKCbeta(2), did not affect LDL uptake, nor did it prevent scavenger receptor A upregulation. Incubation of monocyte-derived macrophages with PMA/LDL increased PKCbeta(1) phosphorylation at the Thr-642 residue, which was blunted by LY379196. However, the expression of CD68, a marker of activated macrophages, was not affected by LY379196. Moreover, LY379196 did not affect lipopolysaccharide-induced CD14 degradation, tumor necrosis factor-alpha release, or superoxide anion production, ruling out any effect of PKCbeta inhibition on innate immunity. CONCLUSIONS: Nonspecific inhibition of PKCbeta prevents LDL uptake in macrophages. These findings suggest that PKCbeta inhibitors may represent a novel class of antiatherosclerotic drugs.

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Inhibition of Protein Kinase Cβ Prevents Foam Cell Formation by Reducing Scavenger Receptor A Expression in Human Macrophages

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Abstract

Background LDL uptake by monocyte-derived macrophages (MDMs) is a crucial step in foam cell formation and early atherosclerotic lesion. Increasing evidence supports that activation of PKCβ is involved in many mechanisms promoting atherosclerosis. Thus, we investigate whether inhibition of PKCβ prevents foam cell formation. ***Methods and Results*** The differentiation of human primary monocytes or monocytic THP-1 cell line into MDMs was induced by phorbol 12-myristate 13-acetate (PMA, 0.1 mmol/L), a potent activator of PKC. Incubation of MDMs with DiI-modified LDL (acLDL and oxLDL, 10 μg/mL) led to lipoprotein uptake. Interestingly enough, the non-selective inhibitor of $PKC\beta_1$ and $PKC\beta_2$, LY379196 ($5x10^{-7}$-10^{-5} mol/L) blunted LDL uptake in MDMs as shown by flow-cytometry. Specific siRNA-mediated knockdown of PKCβ exerted similar effect. Furthermore, PMA alone and in the presence of modified LDL induced scavenger receptor A (SR-A) mRNA and protein expression which was abolished by LY379196. CGP53353, a selective inhibitor of $PKC\beta_2$, did not affect LDL uptake nor did it prevent SR-A upregulation. Incubation of MDMs with PMA/LDL increased $PKC\beta_1$ phosphorylation at the Thr-642 residue, which was blunted by LY379196. However the expression of CD68, a marker of activated macrophages, was not affected by LY379196. Moreover, LY379196 did not affect LPS-induced CD14 degradation, TNFα release nor superoxide anion production, ruling out any effect of PKCβ inhibition on innate immunity. ***Conclusions*** Non-specific inhibition of PKCβ prevents LDL uptake in macrophages. These findings suggest that PKCβ inhibitors may represent a novel class of antiatherosclerotic drugs.

Key Words: PKCβ, low-density lipoproteins, monocyte-derived macrophages, foam cell formation, atherosclerosis.

Protein kinase C (PKC) comprises several structurally related serine/threonine kinases classified in 3 groups. The "conventional" or "classical" PKCs include PKC α, β_1, β_2, γ. These isoforms can be activated by Ca^{2+} and/or by diacylgycerol (DAG) as well as phorbol esters. The "novel" PKC δ, ε, θ are also activated by DAG and phorbol esters but are Ca^{2+} independent. The "atypical" PKCs, which include PKCζ and PKCι, are unresponsive to Ca^{2+}/DAG and phorbol esters.[1] Particularly interesting is the modulation of PKC activity by phosphorylation of serine and threonine amino acid residues within its catalytic and regulatory domains.[2] Both conventional and novel PKC isoforms can traslocate to the membranous compartment of the cell to elicit biological actions in the presence of DAG, of which the de novo synthesis is increased by hyperglycemia.[3] Indeed, the activation of PKC pathway, especially the PKCβ isoform, has been shown extensively to cause diabetic vascular dysfunction.[4-7] As non-selective PKC inhibition is associated with lethal side effects, isoform-specific inhibitors have been developed. The macrocyclic bis (indolyl) maleimides, like ruboxistaurin (LY333531), LY379196, LY317615 and LY290181 are competitive inhibitors of ATP binding site within the PKC molecule.[8,9] The advantage of macrocyclic bis (indolyl) maleimides is their high selectivity for PKCβ in comparison to other isoforms of PKC.[10] Treatment of diabetic patients for 3 months with PKC412, a multitarget kinase inhibitor, which also acts as a nonselective PKC inhibitor, resulted in significant gastrointestinal side effects such as nausea, vomiting and diarrhea as well as liver toxicity.[11] On the contrary, a multicenter, randomized clinical trial with the selective PKCβ inhibitor ruboxistaurin in patients with diabetic retinopathy revealed that the oral administration of this drug was well tolerated and did not cause any adverse events in patients with diabetes at

doses up to 16 mg twice daily for 28 days.[12] Furthermore in patients with type 1 and 2 diabetes and minimal retinopathy, retinal blood flow was increased by the PKCβ inhibitor in a dose-dependent fashion.[13] Treatment for over 1 year with ruboxistaurin has shown beneficial effects in delaying the progression of diabetic nephropathy.[14] These studies provided the first demonstration that a PKCβ isoform-selective inhibitor can be used for long term clinical treatment of diabetic microangiopathy with minimal side effects.[15] However, increasing evidence suggests that activation of PKCβ is involved in many mechanisms promoting atherosclerosis.[16] Interestingly, phorbol 12-myristate 13-acetate (PMA), a structural analogue of DAG - natural activator of PKC - can trigger transformation of monocytes to macrophages.[17] The accelerated atherosclerosis[18] and chronic activation of PKCβ in vascular tissues of diabetic patients, including the retina, heart, aorta and circulating monocytes[4,19,20] prompted us to hypothesize that PKCβ among all PKC isoforms could be involved in foam cell formation.

The present study was designed to investigate whether selective pharmacological inhibition of PKCβ results in prevention of modified LDL uptake and, hence, foam cell formation. Our findings unmask an antiatherosclerotic effect of PKCβ inhibitors extending their application far beyond diabetic vascular complications.

Methods

Cell culture

Peripheral blood mononuclear cells were isolated from healthy control subjects by density centrifugation in BD vacutainer cell preparation tubes with sodium heparin (Becton Dickinson) and further purified by MACS sorting with anti –human CD14 antibody (Miltenyi Biotec, Germany) conjugated with magnetic beads. After density centrifugation, highly purified monocytes were recovered. Human monocyte purity was assessed by flow cytometry (FACSCanto (Becton Dickinson) using FITC-conjugated anti-human CD14 antibody (Miltenyi Biotec). The human monocytic THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Monocytes were cultured in RPMI 1640 medium containing 25 mmol/L Hepes buffer [supplemented with 10% fetal calf serum (FCS), 1% L-glutamine 200 mmol/L, 100 U/mL penicillin, and 100µg/mL streptomycin] in humidified air, 5%CO_2 at 37°C. Freshly isolated human monocytes and THP-1 monocytes were differentiated into MDMs in vitro by treatment with 0.1 µmol/L PMA (Calbiochem, Darmstadt, Germany) for overnight in starvation medium 0.5% FCS RPMI 1640. During starvation the cells were exposed to LY379196 (10^{-6} mol/L), a non-selective inhibitor of both PKCβ isoforms, CGP53353 (10^{-6} mol/L), a PKCβ_2 selective inhibitor and then incubated for further 24 hours in the presence of 10 µg/mL DiI-labeled acetylated low density lipoprotein (DiI-acLDL) and DiI-labeled oxidized low density lipoprotein (DiI-oxLDL) (Intracel, Frederick, MD, USA). Human MDMs were also pretreated with myristoylated cell-permeable myr-ψPKC peptide (10^{-4} mol/L), based on the

pseudosubstrate motif of PKC β which keep the enzyme in an inactive state by interacting with the substrate binding site of PKC β catalytic domain.[21] In another set of experiments MDMs were pretreated with the two PKCβ inhibitors or myr-ψPKC and stimulated for 24 hours with 100 ng/mL, lipopolysaccharide (LPS, Sigma, Buchs, Switzerland). Afterwards TNFα levels were measured in cell supernatant by ELISA (R&D Systems, Minneapolis, MN, USA). To exclude cytotoxicity, a colometric assay for detection of lactate dehydrogenase in cell supernatant was performed according to the manufacturer's recommendations (Roche, Basel, Switzerland).

siRNA transfection

Transfections were performed using INTERFERin TM (Polyplus Transfection, Switzerland) according to the manufacturer's instructions in Human MDMs (THP-1 cell line). Commercially available Human PKCβ, GAPDH (Santa Cruz, Germany) specific siRNAs were used for transfecting. The MDMs were transfected after 24hrs of seeding. 8 µl of INTERFERin transfection reagent (Polyplus) was added to 100 µl of OptiMEM serum-free medium containing 80 nM of each siRNA oligo, incubated for 10 minutes, and added to the 3 cm plate containing 2 ml of medium. GAPDH siRNA was used as a negative control. The control siRNA A – FITC labelled (Santa Cruz, Germany) was used as a marker for transfection efficiency. Gene silencing was measured after 48h by western blotting. The transfected cells were incubated for further 24 hours in the presence of 10 µg/mL DiI-acLDL (Intracel, Frederick, MD, USA) and acLDL uptake was measured by Flow cytometry. Another set of transfected MDMs were used to perform western blotting.

Real Time PCR

Total RNA was extracted from MDMs (THP-1 cell line) using Trizol Reagent (Invitrogen, Basel, Switzerland) according to the manufacturer's recommendations. Conversion of the total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamers (Amersham Bioscience, Otelfingen, Switzerland) in a final volume of 35 µL, using 4 µg of cDNA according to manufacturer recommendations. Real time PCR was performed in a MX3000P PCR cycler (Stratagene, La Jolla, CA). All PCR experiments were performed in triplicates using the SYBR Green JumpStart kit provided by Sigma. Each reaction (25 µL) contained 2 µL cDNA, 1 pmol of each primer, 0.25 µL of internal reference dye and 12.5 µL of JumpStart Taq ReadyMix (containing buffer, dNTPs, stabilizers, SYBR Green, Taq polymerase and JumpStart Taq antibody). The following primers were used. For SR-A: sense primer: 5'-CCAGGGACATGGGAATGCAA–3', antisense primer: 5'–CCAGTGGGACCTCGATCTCC–3'. For human L28: sense primer: 5'–GCATCTGCAATGGATGGT-3', antisense primer: 5'–TGTTCTTGCGGATCATGTGT-3'. The amplification program consisted of 1 cycle at 95°C for 10 min, followed by 40 cycles with a denaturing phase at 95°C for 30 s, an annealing phase of 1 min at 60°C and an elongation phase at 72°C for 1 min. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. For verification of the correct amplification, PCR products were analyzed on an ethidium bromide stained 1% agarose gel. In each real-time PCR run for F3 and L28 a calibration curve was included, that was generated from serial dilutions ($2x10^7$, $2x10^6$, $2x10^5$, $2x10^4$, $2x10^3$, $2x10^3$, copies/reaction for SR-A, and $2x10^7$, $2x10^6$, $2x10^5$, $2x10^4$, $2x10^3$, $2x10^3$ copies/reaction for L28) of purified amplicons for SR-A and L28.

Western blotting

Human MDMs, THP-1 cell line and freshly isolated blood Monocytes (where indicated), were washed twice with PBS and harvested in the extraction buffer (120 mmol/L sodium chloride, 50 mmol/L Tris, 20 mmol/L sodium fluoride, 1 mmol/L benzamidine, 1 mmol/L DTT, 1 mmol/L EDTA, 6 mmol/L EGTA, 15 mmol/L sodium pyrophosphate, 0.8 µg/mL leupeptin, 30 mmol/L *p*-nitrophenyl phosphate, 0.1 mmol/L PMSF, and 1% NP-40) for immunoblotting. All cell debris were removed by centrifugation (12 000 *g*) for 10 minutes at 4°C. The protein extracts (20 µg) were treated with 5x Laemmli's SDS-PAGE sample buffer (0.35 mol/L Tris-Cl, pH 6.8, 15% SDS, 56.5% glycerol, 0.0075% bromophenol blue), followed by heating at 99°C for 5 minutes**,** and then applied to 10% SDS-polyacrylamide gel for electrophoresis. The proteins were then transferred onto Immobilon-P filter papers (Millipore AG, Bedford, MA) with a semidry transfer unit (Hoefer Scientific, San Francisco, CA). The membranes were then blocked by use of 5% skim milk in TBS-Tween buffer (0.1% Tween 20; pH 7.5) for 1 hour and incubated overnight with the anti-human SR-A (1:500, TransGenic, Kobe, Japan), anti-human LOX-1 (1:4000, R&D Systems, Germany), anti-phospho-Thr-642 PKCß$_1$ (1:1000, Biosource, Nivelles, Belgium), anti PKCβ-1 (1:1000, Santa Cruz, Germany), anti-CD14 (1:1000, Dako Cytomation, Baar, Switzerland) antibody in 0.5% BSA PBS. Antigen detection was performed with an enhanced chemiluminescence detection system (Amersham Biosciences, Otelfingen, Switzerland).

LDL uptake measurement by flow cytometry

After incubation with DiI-acLDL (10µg/mL) and DiI-oxLDL (10µg/mL) human MDMs both freshly isolated and THP-1 cell line were subjected to flow cytometry in order to measure the

amount of internalized LDL. Adherent and non adherent cells were harvested by gentle scraping. Cells were then washed twice with PBS and resuspended in 0.2% BSA in PBS. Samples were analyzed using flow cytometer Facscanto II (BD, Heidelberg, Germany) and software "Flowjo".

Measurements of superoxide anion production

Superoxide production in MDMs, both freshly isolated and THP-1 cell line, treated with LY379196 (10^{-6} mol/L) and silencing of PKC β was determined by electron spin resonance (ESR) spectroscopy analysis using the spin trap CM-H (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; Noxygen; Elzach; Germany) as described previously.[22] In brief, MDMs were centrifuged (380 g; 5 min) and resuspended in Krebs-HEPES buffer (pH 7.35) containing diethyldithiocarbamate (DETC; 5μM) and deferoxamine (25μM). ESR spectra were recorded using a Bruker e-scan ESR spectrometer (Bruker Corporation) after the addition of the spin trap CM-H (200μM) under stable temperature conditions (37° C; temperature control system). The ESR instrumental settings were as follows: center field, 3495 G; field sweep width, 10.000 G; microwave frequency, 9.75 GHz; microwave power, 19.91 mW; magnetic field modulation frequency, 86.00 kHz; modulation amplitude, 2.60 G; conversion time, 10.24 msec; detector time constant, 328 msec; number of x-scans, 10.

Confocal Fluorescent Microscopy

Human MDMs were washed once with PBS, fixed in 4% paraformaldehyde for 10 min, washed again with PBS, blocked with 0.1 mol/L glycine for 5 min, permeabilized with 0.2% Triton 100 for 7 min and incubated overnight with anti-human SR-A (TransGenic, Kobe, Japan), anti-PKCß$_1$ (Biosource, Nivelles, Belgium), anti-PKCß$_2$ (Biosource, Nivelles, Belgium) or anti-CD68 (Dako

Cytomation, Baar, Switzerland) antibodies in 0.2% BSA. Afterwards the cells were washed three times with PBS and were incubated with secondary alexa fluor488-labeled antibody (Molecular Probes, Eugene, OR) in 0.2% BSA for 1 hour. Cells were counterstained with 4', 6 diamidino-2-phenylindol (DAPI, Vector Laboratories, Burlingame, CA) and analyzed using Leica confocal laser microscope.

Drugs

LY379196 was provided by Eli Lilly (Indianapolis, IA, USA). CGP53353 was kindly provided by Dr. Doriano Fabbro (Novartis Pharma AG, Basel, Switzerland). Calphostin C, PMA and myr-ψPKC were purchased from Calbiochem (Darmstadt, Germany). LPS was obtained from Sigma (Buchs, Switzerland).

Statistical Analysis

Results are expressed as mean±SEM and n indicates number of experiments. Statistical evaluation of the data was performed with Student's *t* test for simple comparison between 2 values when appropriate. For multiple comparisons results were analyzed by ANOVA followed by Fisher's test. A value of $p<0.05$ was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Role of PKCβ in mediating human MDM foam cell formation

The differentation of human primary monocytes and monocytic THP-1 cell line into macrophages (MDMs) was induced by PMA (0.1µmol/L). Incubation of human MDMs with DiI-acLDL (10 µg/mL) led to binding of acLDL to the plasma membrane and accumulation of lipoproteins into the cytoplasm as assessed by fluorescence microscopy (Figure 1A). The non-selective inhibitor of both PKCβ isoforms LY379196 (10^{-6}mol/L) abolished acLDL uptake in MDMs as shown in Figure 1B. To identify the isoform of PKCβ involved, human MDMs were exposed to selective antibodies against $PKC\beta_1$ and $PKC\beta_2$. PKCβ isoforms were localized in the nucleus as well as in cytoplasm of MDMs, showing an activated state of the enzyme. $PKC\beta_1$ isoform was homogeneously distributed within the cytoplasm and plasma membrane (Figure 1C), while $PKC\beta_2$ was present in the perinuclear patch and plasma membrane (Figure 1D).

Accordingly, LY379196 showed a dose-dependent decrease of both acLDL and oxLDL uptake in MDMs by flow-cytometry (Figure 2A and B). In contrast, selective inhibitor of $PKC\beta_2$ CGP53353 did not exert any significant effect (data not shown).

Effect of PKCβ inhibition on SR-A expression

To delineate the molecular mechanism by which LY379196 blunted modified LDL uptake MDMs gene and protein expression of scavenger receptor A (SR-A) were determined. Quiescent

cells did not express SR-A mRNA, stimulation with PMA (0.1μmol/L) increased SR-A expression (Figure 3). A more pronounced upregulation of SR-A expression was achieved by addition of acLDL (10 μg/mL) to the medium. SR-A mRNA expression was abolished by treatment with LY379196 (10^{-6} mol/L), whereas CGP53353 (10^{-6} mol/L) did not exert any inhibitory effect on PMA/acLDL-induced SR-A expression in human MDMs (Figure 3). In agreement with these results, SR-A protein expression was increased after exposure to acLDL as well as oxLDL (Figure 4A and B). Treatment with LY379196, but not with CGP53353, totally abrogated PMA/acLDL-induced macrophage SR-A expression (Figure 4A). When MDMs were exposed to oxLDL in the presence of LY379196 (10^{-6}mol/L) showed similar results (Figure 4B). A myristoylated cell-permeable myr-ψPKC inhibitory peptide was used to confirm the modulatory role of PKCβ on SR-A expression. As LY379196, the inhibitory peptide myr-ψPKC (10^{-4}mol/L) significantly blunted SR-A protein expression in human MDMs (Figure 4C).

In contrast to SR-A, LOX-1 protein expression did not change after exposure of MDMs with modified LDL. Furthermore, LY379196 (10^{-6}mol/L) did not exert any significant effect on LOX-1 expression (Figure 4D).

Effect of silencing PKCβ on LDL uptake and SR-A expression

The transfection of PKCβ siRNA into MDMs resulted in reduced expression of the protein, whereas the GAPDH and Mock controls did not exert any significant effect (Figure 5A). We examined with flow cytometry the DI-acLDL uptake by the transfected cells. PKCβ silencing reduced the uptake and blunted SR-A protein expression, reproducing the same effect as that of the pharmacological inhibitor LY379196 (Figure 5B and C).

Role of Thr-642 phosphorylation in PKCβ_1-mediated SR-A Expression

Western blotting with an antibody against phosphorylated PKCβ_1 at specific amino residue revealed that incubation of the cells with PMA/acLDL increased Thr-642 phosphorylation within the catalytic domain of this molecule (Figure 6). The inhibitor of both PKCβ isoforms, LY379196 (10^{-6} mol/L) as well as inhibitory peptide myr-ψPKC (10^{-4}mol/L) blunted PMA/acLDL-induced Thr-642 phosphorylation (Figure 6), suggesting that phopsphorylation of PKCβ_1 at Thr-642 may thus represent a selective regulatory mechanism for SR-A upregulation. Accordingly, the selective PKCβ_2 inhibitor CGP53353 (10^{-6} mol/L) did not affect Thr-642 phosphorylation (Figure 6).

Effect of PKCβ inhibition on Macrophage Activation and Functioning

As shown by confocal microscopy, high levels of SR-A expression in MDMs stimulated with acLDL were blunted in the presence of LY379196 (Figure 7A and B). However, LY379196 did not exert any effect on the expression of CD68, a marker of macrophage activation, (Figure 7 C and D). In addition, human MDMs were exposed to LPS (100 ng/mL) to rule out an effect of PKCβ inhibition on macrophage functioning in innate immunity. LPS elicited degradation of CD14 and secretion of TNFα, crucial steps in the activation of innate immunity (Figure 8A and B). Interestingly enough, increasing concentrations of LY379196 did not inhibit neither LPS-induced CD14 degradation (Figure 8A) nor TNFα release (Figure 8B). Treatment with LY379196 did not elicit any release of LDH (data not shown), ruling out that its effects on human MDMs were due to cellular toxicity. In agreement with this finding, no apoptotic nuclei

by DAPI staining were observed (Figure 7). Furthermore, effect of LY379196 (10^{-6} mol/L) on the ability of macrophages to produce superoxide anion (O_2^-) was measured. No significant changes in O_2^- production were observed (Figure 8C). Also silencing of PKCβ did not affect O_2^- production (data not shown).

Discussion

Accumulation of cholesterol-loaded foam cells in the arterial intima is a hallmark and key event of early atherogenesis.[23] Circulating monocytes adhere to activated endothelial cells and transmigrate into the subintima to become tissue macrophages. Upon exposure to modified lipoproteins such as the oxidized and acetylated forms of low-density lipoproteins (oxLDL and acLDL), these macrophages become foam cells.[24] Two receptors appear to be essential in foam cell formation and receptor-mediated binding/ uptake of modified lipoproteins: CD36 and SR-A.[25] Despite increasing evidence supports that PKC is involved in many mechanisms promoting atherosclerosis,[16] only few studies have examined the role of PKC signaling in foam cell formation.[20, 26]

In this study we demonstrated that inhibition of PKCβ prevents uptake of modified LDL by reducing human MDMs SR-A expression. Several lines of evidence support this conclusion. First, FACS analysis revealed that the non-selective inhibitor of PKCβ isoforms blunted modified LDL uptake of human MDMs. Second, silencing of PKCβ by siRNA transfection also reduced LDL uptake. Third, we observed a selective Thr-642 phosphorylation within the catalytic domain of $PKC\beta_1$ as well as increase of SR-A mRNA and protein expression in human MDMs exposed to modified LDL. Fourth, both LY379196 and inhibitory peptide myr-ψPKC blunted phosphorylation of Thr-642 and upregulation of SR-A. By contrast CGP53353, the selective inhibitor of $PKC\beta_2$.[7] did not exert any significant effect.

Expression and function of macrophage SR-A plays a crucial role in the pathogenesis of atherosclerosis.[27,28] Accordingly, SR-A gene deficient mice bred with atherosclerosis-prone

apoE-/- or *LDLrec-/-* mice have been found to develop less atherosclerosis.[29] We recently showed that *ApoE-/-* mice simultaneously lacking c-Jun N-terminal kinase 2 (*ApoE-/- JNK2-/-* mice) developed less atherosclerosis than do *ApoE-/-* mice.[30] Macrophages lacking JNK-2 displayed markedly decreased phosphorylation of SR-A and, hence, suppressed foam cell formation.[30] Thus, upstream signaling molecules that regulate expression and function of SR-A may represent potential targets for therapeutic interventions.[24] The present findings clearly indicates that SR-A expression in human MDMs is regulated by PKCβ. In contrast to SR-A, LOX-1 expression did not change after stimulation of MDMs with modified LDL. Furthermore, LY379196 did not exert any significant effect on LOX-1. Since SR-A expression was enhanced upon modified LDL stimulation and not LOX-1 we conclude that SR-A might be the primary receptor for modified LDL uptake.

Several studies have strongly implicated activation of PKCβ in the pathogenesis of the vascular complications of diabetes.[31] The synthesis of isoform-specific inhibitors for PKCβ has provided not only important insights into diabetic cardiovascular disease but also effective drugs against diabetic microvascular complications.[15,32,33] Glucose-induced activation of PKCβ may lead to endothelial dysfunction by causing activation of vascular NADPH oxidase, eNOS uncoupling and ROS production.[6,34] Furthermore, high glucose enhances human macrophage LOX-1 expression via PKCβ activation.[20] Treatment with PKCβ inhibitor prevents impaired endothelium-dependent vasodilation caused by hyperglycemia.[5] We demonstrated that selective inhibition of $PKC\beta_2$ inhibits glucose-induced VCAM-1 expression in human endothelial cells.[7] Interestingly enough, our data unmasks an antiatherosclerotic effect of PKCβ inhibitors even in the nondiabetic condition of hypercholesterolemia. Specific siRNA-mediated knockdown of

PKCβ further supports our conclusion. Indeed, upon silencing of PKCβ the LDL uptake was blunted, SR-A expression reduced and, hence, foam cell formation prevented.

The molecular link between PKCβ signaling pathway, SR-A upregulation and uptake of modified LDL might involve Thr-642 phosphorylation within catalytic domain of $PKC\beta_1$. Indeed, inhibition of PKCβ either by LY379196 or inhibitory peptide myr-ψPKC blunting PMA/acLDL-induced Thr-642 phosphorylation abolished upregulation of SR-A as well as LDL uptake of human MDMs. In contrast, selective inhibitor of $PKC\beta_2$ CGP53353 did not affect any of these events. According to our results phosphorylation of $PKC\beta_1$ at Thr-642 represents a selective regulatory mechanism for SR-A upregulation and foam cell formation.

Of particular interest is the fact that in our study LY379196, as a drug targeting macrophages, prevents only foam cell formation without affecting macrophage host-defense activity. Indeed, LY379196 blunted modified LDL uptake but did not affect the expression of CD68, a marker of macrophage activation.[35] We also demonstrated that LY379196 did not inhibit LPS-induced CD14 degradation nor TNFα release in human MDMs. Moreover, PKCβ knockdown or inhibition did not affect superoxide anion production. These findings rules out any effect of PKCβ inhibition on macrophage functioning in innate immunity. In agreement with our results PKCβ-deficient mice have not been reported to present any impairment of macrophage activity.[36] Although these mice show a reduced peritoneal population of B-1 lymphocytes, the absolute number of splenic B cells is similar to the wild type animals. Furthermore, the thymuses of PKCβ-deficient mice were of normal size and cellularity and contained $CD4^+CD8^+$ double-positive cells and $CD4^+$ or $CD8^+$ single-positive cells at normal ratios.[36]

Earlier studies of the role of vascular PKCβ activation in diabetes were primarily focused on microvascular dysfunction.[37,38] Indeed, PKCβ inhibitors are currently being tested in clinical trials with microvascular endpoints.[12-14] In conclusion, the results of our study suggest a role for PKCβ in atherogenesis even in the nondiabetic condition and anticipate the application of PKCβ inhibitors as putative antiatherosclerotic drugs. However, before deciding whether PKCβ inhibitors deserve to be tested in clinical trials of atherosclerosis, animal models will help evolve our current suggestive in vitro evidence concerning pro-atherosclerotic role of PKCβ signaling.

Founding Sources

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Disclosures

There is no potential conflict of interest.

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Figure Legend

Figure 1. Fluorescent confocal microscopy of human MDMs after incubation with DiI-acLDL (A). Red particles in the cytoplasm represent internalized DiI-acLDL. LY379196 ($5x10^{-6}$ mol/L) abolished modified LDL uptake (B). In human MDMs, green staining shows intracellular distribution of PKCβ_1 (C) and PKCβ_2 (D). Staining for nuclei in blue with DAPI.

Figure 2. FACS analysis of human MDMs in the presence or in the absence of LY379196 after 24 hours incubation with DiI-acLDL (A) and DiI-oxLDL (B). LY379196 ($5x10^{-6}$ mol/L) blunted modified LDL uptake, blue color. CGP53353 did not shown any significant effect (data not shown). LY379196 exerted a dose-dependent inhibition of acLDL and oxLDL uptake (A and B, respectively). Results are presented as mean±SEM; n=4 in each group. *$p<0.05$ vs PMA plus acLDL or oxLDL, respectively.

Figure 3. Expression of Scavenger Receptor-A (SR-A) in PMA-induced human MDMs as well as after incubation with acLDL in the presence and in the absence of LY379196 (10^{-6} mol/L) or CGP53353 (10^{-6} mol/L). SR-A mRNA expression assessed by real-time PCR is normalized to L28 mRNA. Results are presented as mean±SEM; n=3 in each group. *$p<0.05$ vs control, **$p<0.05$ vs PMA alone, $^{\dagger}p<0.05$ vs PMA plus acLDL.

Figure 4. Representative Western blot and densitometric quantification of SR-A protein expression in PMA-induced human MDMs as well as after incubation with acLDL (A) and oxLDL (B) in the presence and in the absence of LY379196 (10^{-6} mol/L) or CGP53353 (10^{-6} mol/L). Inhibitory effect of myristoylated cell-permeable peptide, myr-ψPKC on SR-A protein expression in PMA-induced human MDMs after incubation with acLDL (C). Lectin-like Oxidized low density lipoprotein receptor 1 (LOX-1) protein expression in PMA-induced human MDMs as well as after incubation with acLDL and oxLDL in the presence and in the absence of LY379196 (D). Results are presented as mean±SEM; n=4 in each group. *p<0.05 vs control, **p<0.05 vs PMA alone, †p<0.05 vs PMA plus acLDL or oxLDL, respectively.

Figure 5. Representative Western blot and densitometric quantification of PKCβ (A) and SR-A (C) protein expression after transfection of selective PKCβ siRNA into MDMs. FACS analysis of transfected human MDMs after 24 hours incubation with DiI-acLDL (B). Upon silencing of PKCβ the uptake of modified LDL and SR-A expression are reduced . GAPDH and Mock served as controls. Results are presented as mean±SEM; n=4 in each group. *p<0.05 vs Mock and GAPDH, respectively.

Figure 6. Representative Western blot and densitometric quantification of PKCβ1 phosphorylation at the Thr-642 residue in PMA-induced human MDMs after incubation with acLDL in the presence and in the absence of LY379196 (10^{-6} mol/L), CGP53353 (10^{-6} mol/L) or myr-ψPKC (10^{-4} mol/L). Results are presented as

mean±SEM; n=4 in each group. *p<0.05 vs control, **p<0.05 vs PMA alone, †p<0.05 vs PMA plus acLDL.

Figure 7. Treatment with LY379196 (10^{-6} mol/L) affects SR-A expression but not activation of human MDMs stimulated with acLDL. SR-A (A and B) and CD68, a marker of macrophage activation, (C and D) are shown by fluorescent confocal microscopy. The nuclei stained with DAPI are blue in colour, whereas SR-A and CD68 stainings are both green.

Figure 8. LY379196 did not inhibit LPS-induced CD14 degradation, TNFα release nor superoxide anion (O_2^-) production in human MDMs. Representative Western blot and densitometric quantification of CD14 expression (A), TNFα levels in supernatant (B) after 24 hours stimulation with LPS (100ng/mL). O_2^- production after incubation with acLDL and oxLDL (10 μg/mL, C). Results are presented as mean±SEM; n=3-5 in each group.

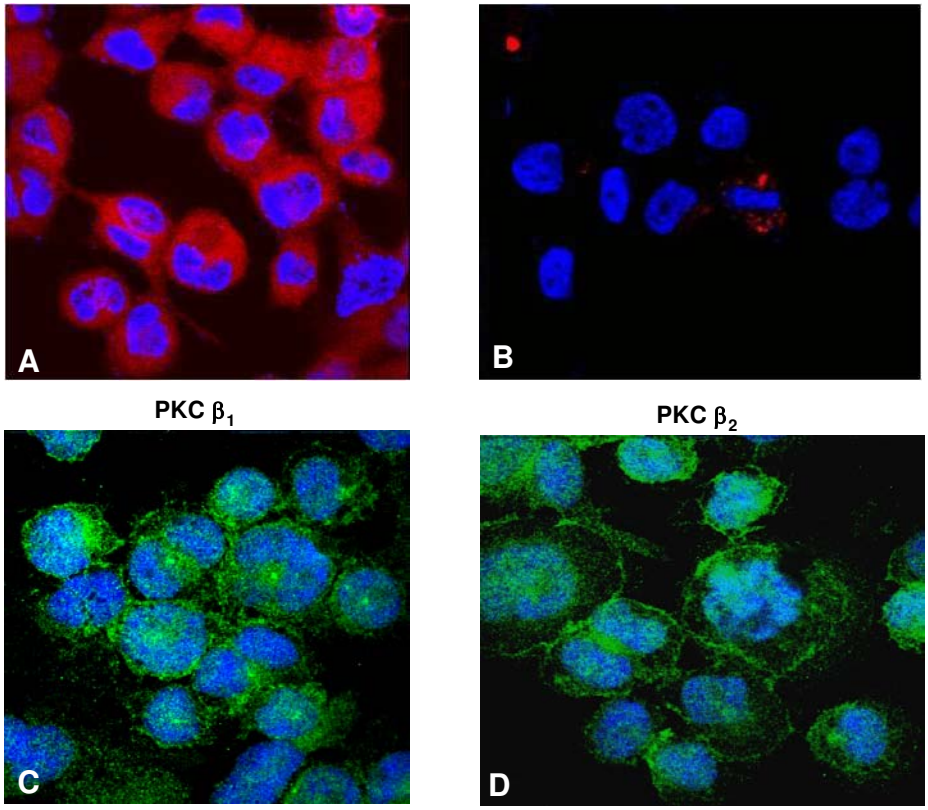

Figure 1

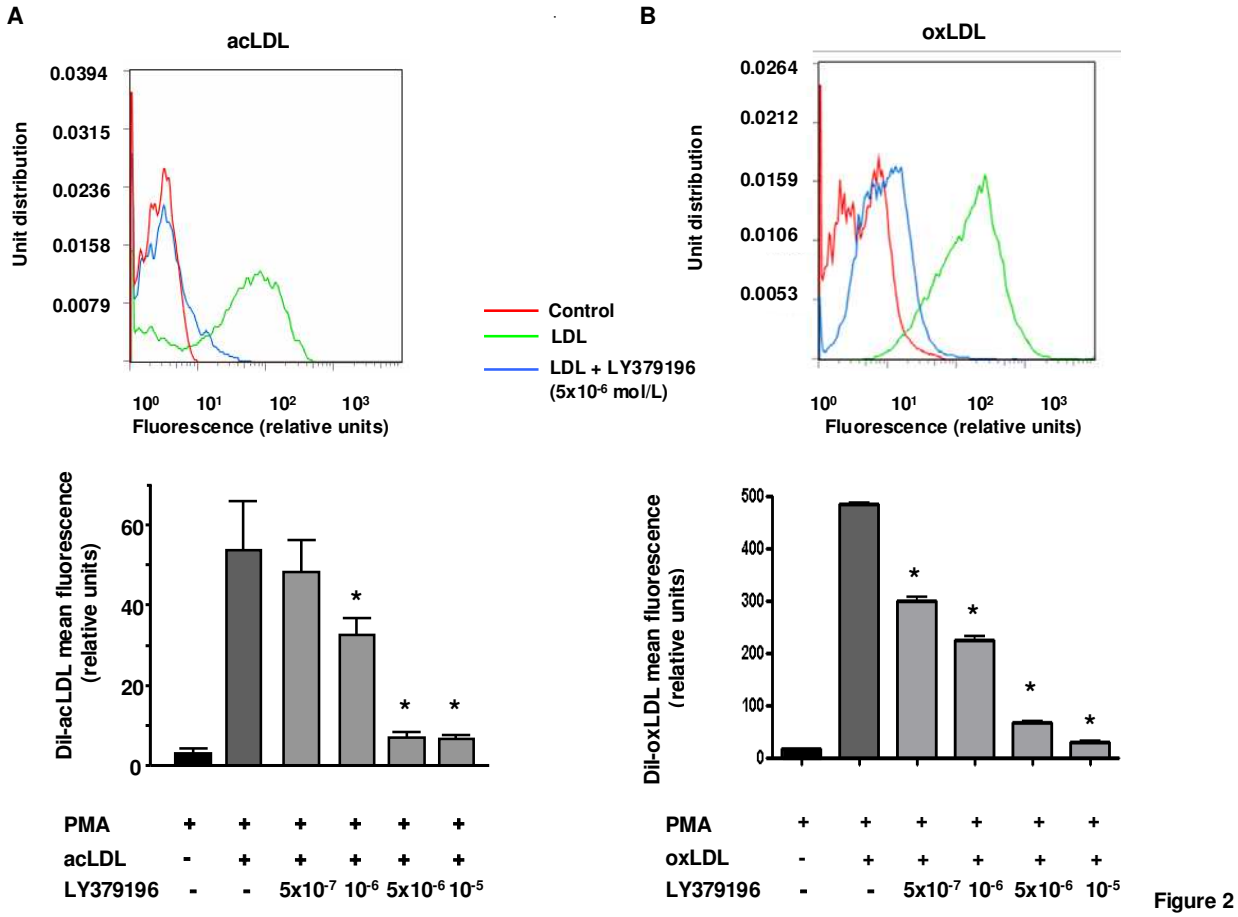
A
acLDL
Unit distribution
0.0394
0.0315
0.0236
0.0158
0.0079
10⁰ 10¹ 10² 10³
Fluorescence (relative units)
Control
LDL
LDL + LY379196
(5x10⁻⁶ mol/L)
B
oxLDL
Unit distribution
0.0264
0.0212
0.0159
0.0106
0.0053
10⁰ 10¹ 10² 10³
Fluorescence (relative units)
DiI-acLDL mean fluorescence
(relative units)
60
40
20
0
PMA + + + + + +
acLDL - + + + + +
LY379196 - - 5x10⁻⁷ 10⁻⁶ 5x10⁻⁶ 10⁻⁵
DiI-oxLDL mean fluorescence
(relative units)
500
400
300
200
100
0
PMA + + + + + +
oxLDL - + + + + +
LY379196 - - 5x10⁻⁷ 10⁻⁶ 5x10⁻⁶ 10⁻⁵

Figure 2

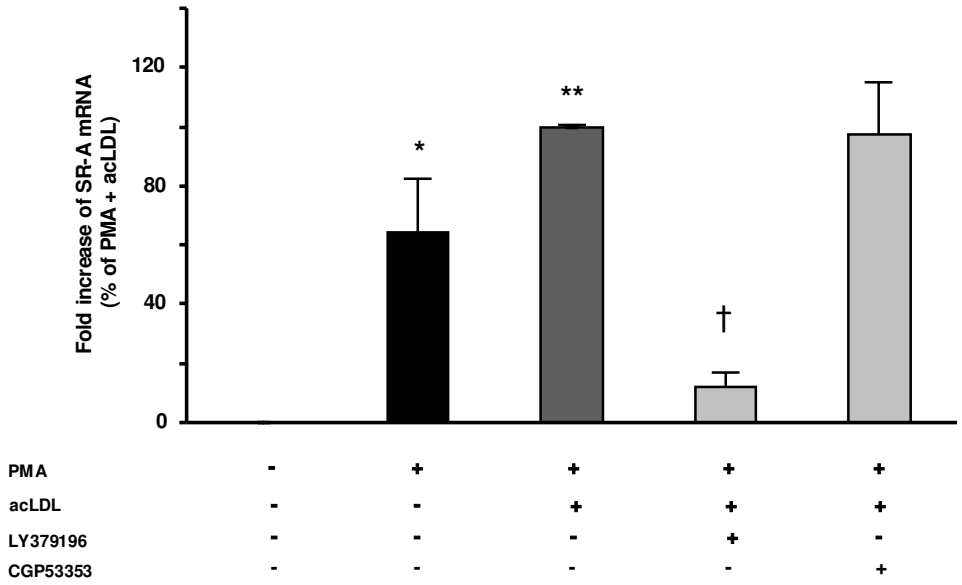
Fold increase of SR-A mRNA
(% of PMA + acLDL)
120
80
40
0
*
**
†
PMA - + + + +
acLDL - - + + +
LY379196 - - - + -
CGP53353 - - - - +

Figure 3

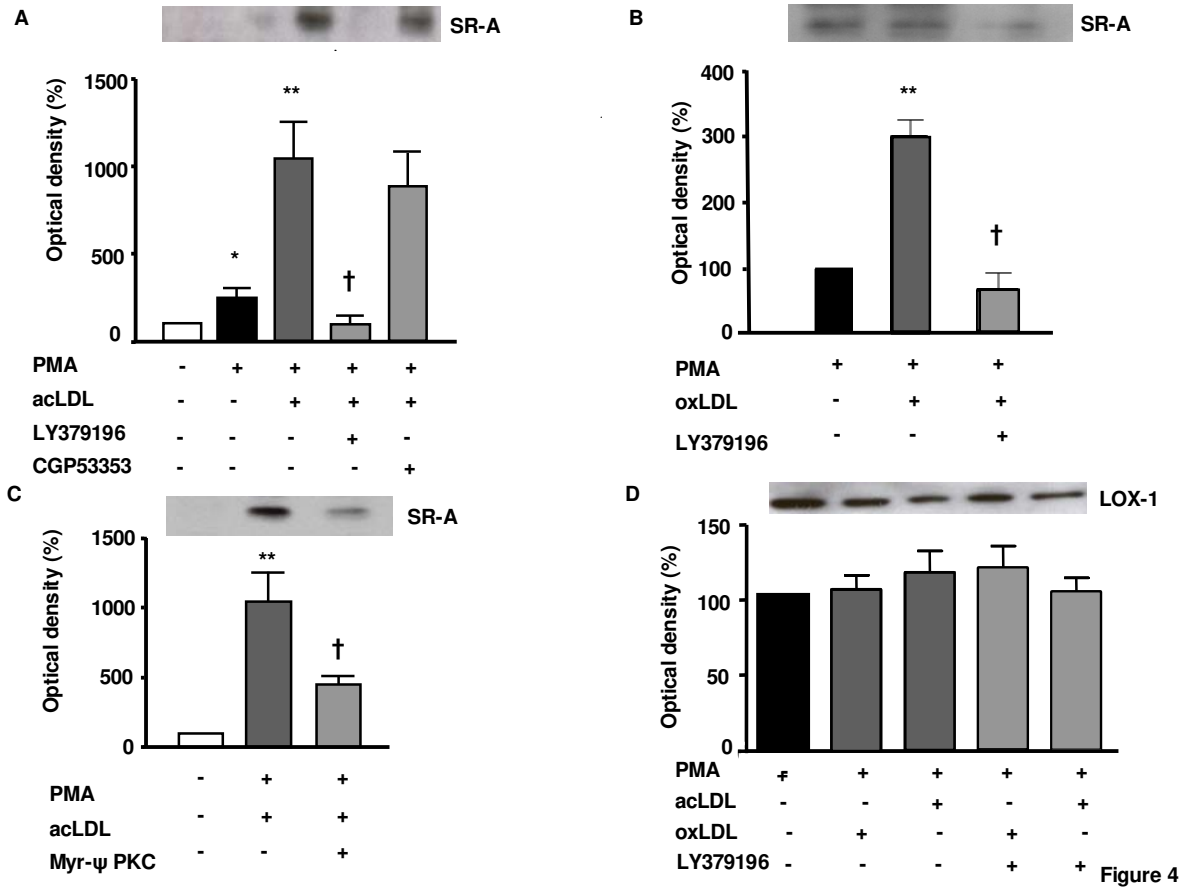
A
SR-A
Optical density (%)
1500
1000
500
0
*
**
†
PMA - + + + +
acLDL - - + + +
LY379196 - - - + -
CGP53353 - - - - +
B
SR-A
Optical density (%)
400
300
200
100
0
**
†
PMA + + +
oxLDL - + +
LY379196 - - +
C
SR-A
Optical density (%)
1500
1000
500
0
**
†
- + +
PMA
- + +
acLDL
- - +
Myr-ψ PKC
D
LOX-1
Optical density (%)
150
100
50
0
PMA + + + + +
acLDL - - + - +
oxLDL - + - + -
LY379196 - - - + +

Figure 4

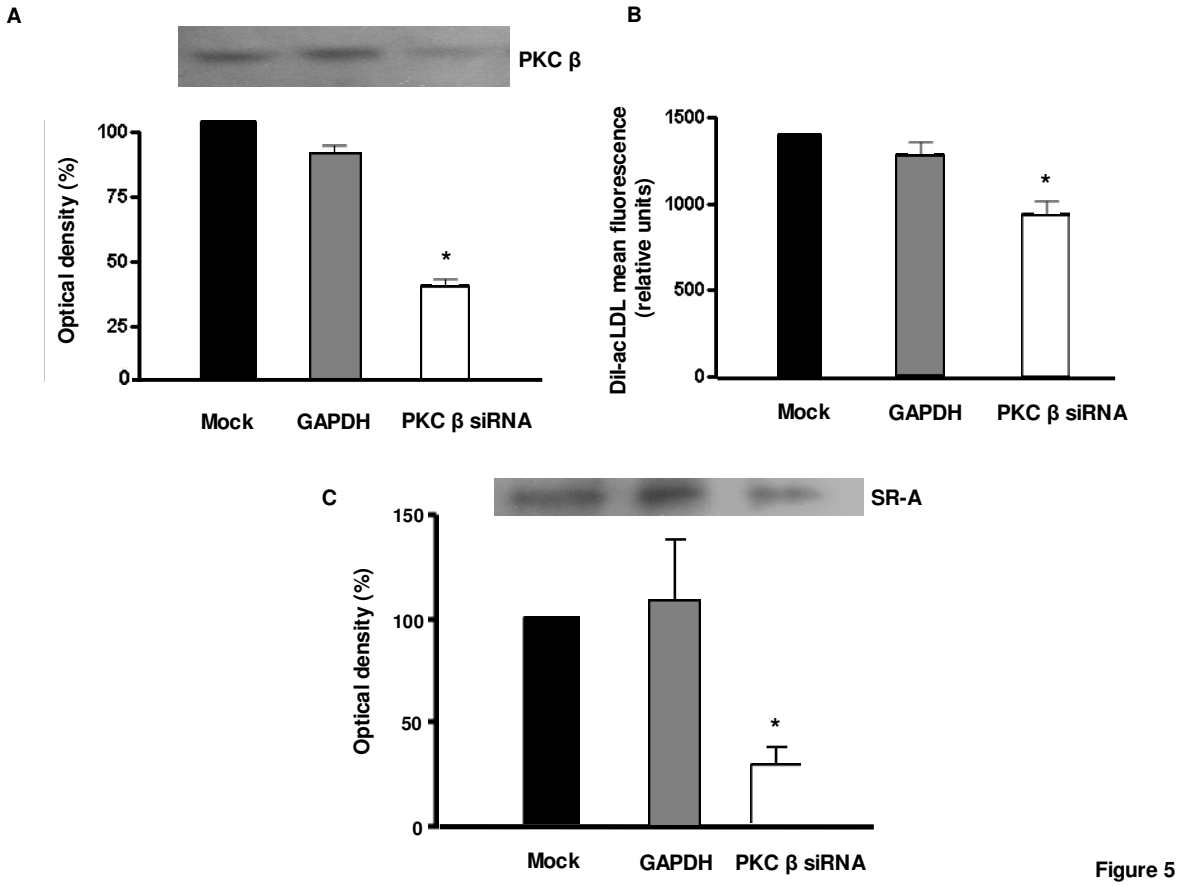
A
PKC β
Optical density (%)
100
75
50
25
0
*
Mock
GAPDH
PKC β siRNA
B
Dil-acLDL mean fluorescence (relative units)
1500
1000
500
0
*
Mock
GAPDH
PKC β siRNA
C
SR-A
Optical density (%)
150
100
50
0
*
Mock
GAPDH
PKC β siRNA

Figure 5

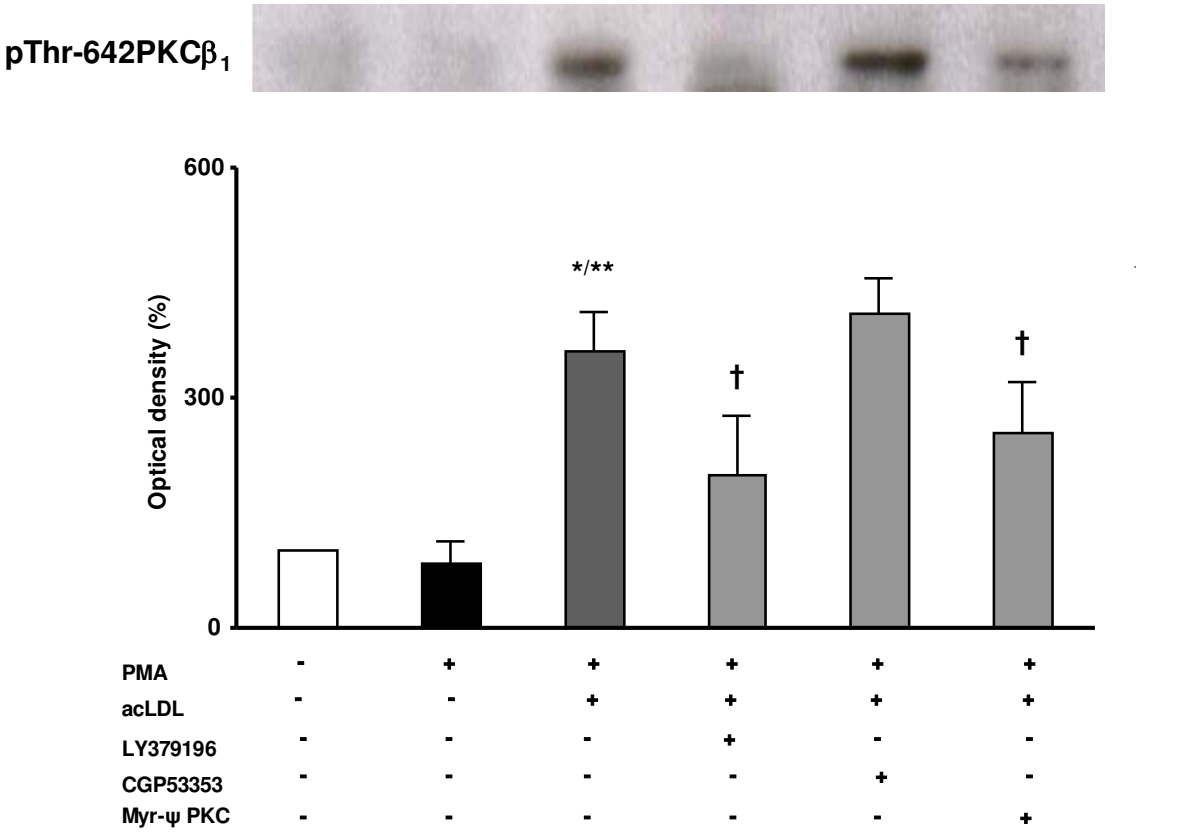
pThr-642PKCβ1
600
300
0
Optical density (%)
*/**
†
†
PMA - + + + + +
acLDL - - + + + +
LY379196 - - - + - -
CGP53353 - - - - + -
Myr-ψ PKC - - - - - +

Figure 6

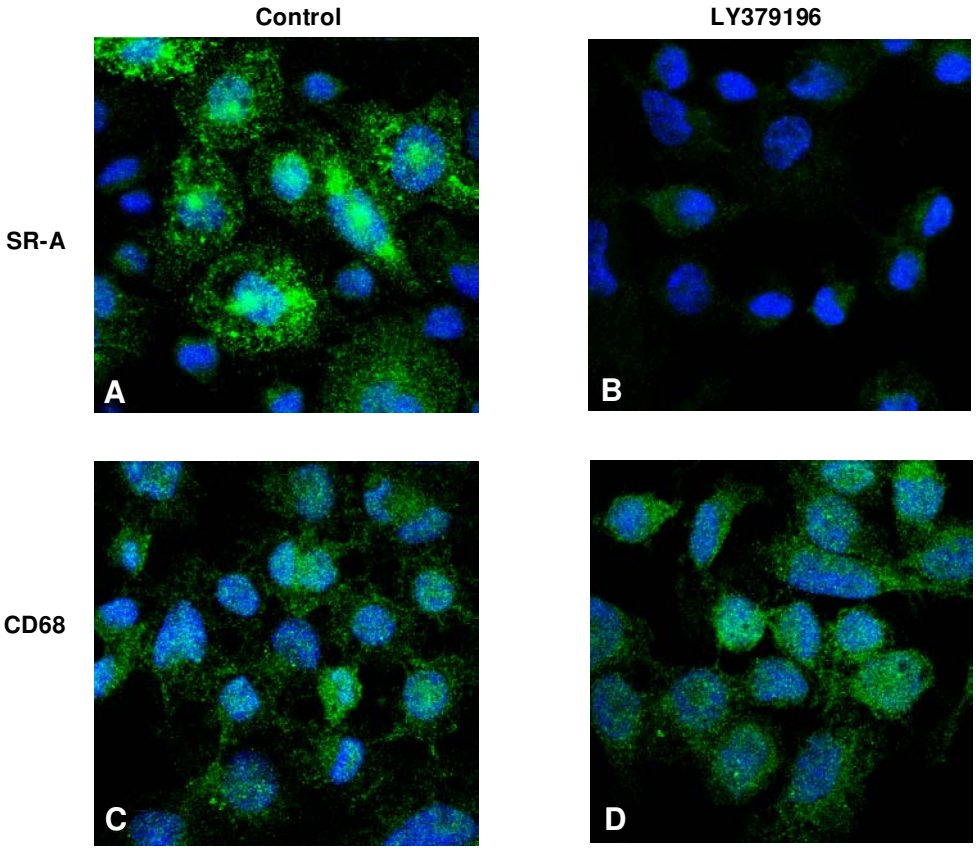

Figure 7

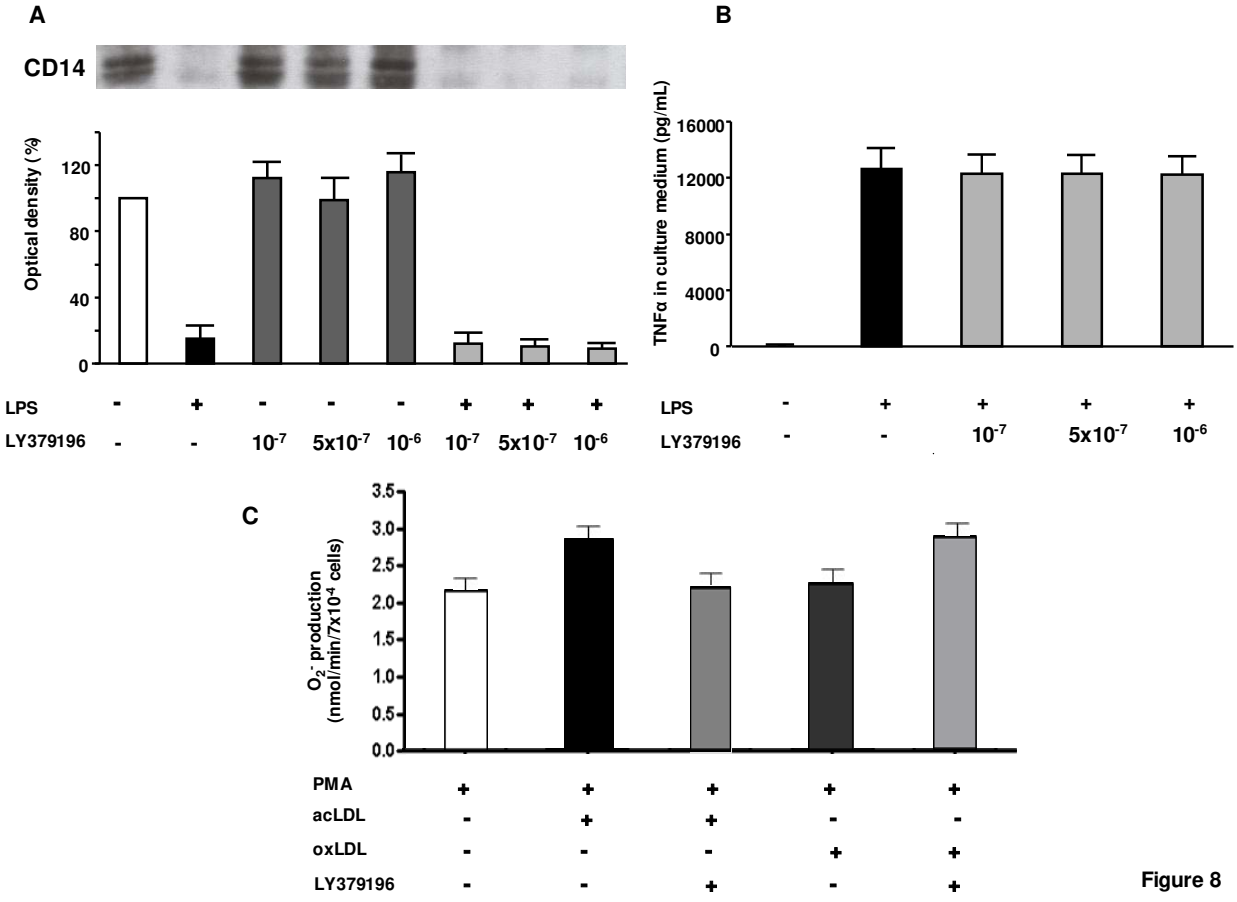
A
CD14
Optical density (%)
120
80
40
0
LPS - + - - - + + +
LY379196 - - 10-7 5x10-7 10-6 10-7 5x10-7 10-6
B
TNFα in culture medium (pg/mL)
16000
12000
8000
4000
0
LPS - + + + +
LY379196 - - 10-7 5x10-7 10-6
C
O2- production (nmol/min/7x10-4 cells)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PMA + + + + +
acLDL - + + - -
oxLDL - - - + +
LY379196 - - + - +

Figure 8